

THE PHYSIOLOGY OF EXCRETION IN MOLGULA (TUNICATA, ASCIDIACEA)

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INTRODUCTION

Twelve years ago (Das, 1936) the author wrote: "The excretory function in ascidians is attributed to a number of organs, the renal nature of some of which is still imperfectly understood. The refringent organ of the Botryllidae, the pyloric gland of the Cynthiidae, the renal vesicles of the Molgulidae and Ascidiidae, the parietal vesicles of the Cynthiidae (Pyuridae), and the neural gland of all ascidians, have been described by different workers as excretory organs." Although our knowledge of the subject has advanced considerably due to the contributions of Azema (1926, 1928, and 1929) and George (1936), so far as the fundamentals of the subject are concerned the above mentioned remarks still hold true.

The present investigation was taken up by the author during his stay in Woods Hole, in the summer of 1947, with a view to elucidating the structure, nature and relationships of the excretory organs and their products in *Molgula manhattensis* DeKay.

Lacaze-Duthiers (1874, 1892) was among the very first to locate and describe the renal organs in *Tunicata* including the renal sacs in Pyuridae (Cynthiidae). Roule (1885) described renal organs in Phallusidae, while Herdman (1888, 1899) described excretory organs in Ascidiidae, Botryllidae, and other ascidians; Dahlgrün (1901) worked on the excretory organs of Botryllidae and Ascidiidae, and compiled an account of excretory organs in *Tunicata*. Das (1936) gave an account of the excretory organs in *Herdmania*, while George (1936) described blood cells bearing excretory matter in *Tunicata*.

Küpffer (1872, 1874) was the first to demonstrate uric acid in *Tunicata*. Sulima (1914) attacked the problem of physiology of excretion in ascidians and showed that uric acid and other purine bases were the chief constituents of the excretory products. Schmidt (1924) confirmed the presence of birefringent purine derivative granules in the renal concretions of ascidians. Millot (1923) extended the investigation to other ascidians, and not only found purine bases as the chief excretory product, but discovered that in Pyuridae (Cynthiidae) the excretory granules are always made up of xanthine. Azema (1926-29) in a series of papers dealt with the mechanism of excretion in some ascidians, and demonstrated the presence of purine bases as granules inside certain blood and connective tissue cells. Finally, George (1936) has shown that certain blood cells have refractory granules of an excretory nature, and that these may circulate freely in the blood stream or be localized in the connective tissue.

From our present knowledge of the subject, therefore, *Tunicata* can be divided into two groups, based on the presence or absence of definite renal vesicles or sacs

which not only filter the nitrogenous excretory products from the blood, but also store them in a more or less insoluble form as solid renal concretions. These two groups of *Tunicata* may be called :

- (a) *the renal type*—with a definitive renal sac, and
- (b) *the arenal type*—without any such sac.

By far the greater number of tunicate species belong to the arenal type, the renal species being mainly confined to the Ascidiidae, the Molgulidae and the Pyuridae.

In the arenal tunicates, the renal function is performed by the renal cells of the connective tissue and blood, as well as the central part of the neural gland (George, 1936; Das, 1936). The localization of the excretory granules may take place in the walls of the vas deferens (*Ciona*; Roule, 1885); the mesenchyme meshwork between oesophagus and stomach on the one hand and rectum on the other (Botryllidae; Dahlgrün, 1901); the immediate neighborhood of the gut (*Ciona intestinalis*; Dahlgrün, 1901); the white pigmented patches on siphons (*Ascidia pellucida*; Azema, 1929b).

The renal tunicates, on the other hand, have a large well-marked vesicle (Molgulidae; Roule, Lacaze-Duthiers) or a number of small vesicles or sacs arranged inside the body wall or in other regions of the body (Ascidiidae and Pyuridae; Lacaze-Duthiers, Dahlgrün, and Azema). These multicellular, blind, ductless, renal sacs not only store a fluid comparable with the urine of other chordates, but strangely enough continuously deposit inside them solid concretions that accumulate throughout life (Azema, 1926).

It is remarkable that in the same family we have some species with definite renal vesicles and others which have only scattered renal cells; e.g. in the family Pyuridae, Dahlgrün (1901) records renal vesicles in *Cynthia dura* and *Microcosmus scrotus*, while Azema (1929a) found no renal vesicles in a number of Pyurid species, but renal cells were present. Similarly George (1936) could find no renal sacs in *Pyura vittata* although renal cells were again present. Das (1936) could find no renal sacs in *Herdmania pallida* (Pyuridae) but found renal cells with refractory granules not only in the visceral region, but also being discharged into the lumen of the neural gland, whence they pass out through the neural duct into the branchial cavity and thence to the outside. According to the present state of our knowledge it is highly improbable, however, that individuals of the same species may possess both the renal vesicles as well as the localized renal cells, although scattered renal cells may often be found.

MATERIAL AND METHODS

The renal vesicle in the Molgulidae is a large bean-shaped sac in which waste matter is gradually deposited and stored in the form of concretions throughout the life of the animal. *Molgula manhattensis* was selected because of its abundance in the Woods Hole region, and because it is comparatively easy to expose or isolate the renal sac in the living condition. The structure of the renal sac was investigated both in the living and the fixed material, while the renal concretions were examined under reflecting as well as polarizing microscopes.

The renal fluid was extracted from living renal sacs by means of a specially made micro-pipette. Care was taken to store the renal fluid under a layer of heavy

neutral mineral oil, and the method of Walker (1930 and 1937) used to determine the total molecular concentrations. The correlations between body weights and weights of renal vesicles were obtained by carefully weighing the entire live animal as well as the freshly isolated renal vesicles under standard conditions. The renal concretions were carefully separated from each vesicle, dried for a half hour at constant temperature, and weighed quickly when each batch was ready.

STRUCTURE OF THE EXCRETORY ORGANS

The main excretory organ in *M. manhattensis* is the large, blind, and ductless renal sac situated on the right side of the body, attached to the body wall or mantle just below the pericardium. It is a thin-walled, elongated, bean-shaped sac and contains a light, yellowish-brown fluid together with the solid concretions deposited in it. It is firmly attached to the wall of the mantle and is in such close proximity to the pericardial wall anteriorly, that it is difficult to separate the renal vesicle without destroying the heart. With each wave of contraction of the heart, the wall of the pericardium is pressed against the antero-dorsal wall of the renal sac, so that the waves of contraction appear to move over this part of the sac as a series of half-ring contractions.

The renal sac lies at about two-thirds distance from the antero-dorsal border of the animal and about one-third from the postero-ventral. It lies across the mantle occupying about one third of its width at its middle (Fig. 1). In a full grown animal measuring $4 \times 3 \times 1.5$ cm., the sac is about 16 mm. long and about 6 mm. at its greatest width. One end of the sac is bluntly rounded while the other is tapering and bent, appearing like an elongated bean in outline (Fig. 2). It is circular in cross-section in the fresh condition but tends to become flattened from side to side in fixed material (Fig. 3). In very young specimens the length of the sac is comparatively short and it appears perfectly bean-shaped; but as the animals increase in size, the sac increases more in length than in width.

The wall of the sac consists of a single layer of cells (one cell thick) which is bounded by a thin basement membrane. Each cell is almost like a cubical epithelial cell although the width is greater than the height. The nucleus lies nearer the basal end of the cell, while the cytoplasm contains one, two, or more vacuoles of different sizes (Fig. 4). The structure of each cell was best seen in the living condition, when a light methylene blue intra-vitam stain was used. The largest vacuole lying on the distal periphery of the cell always takes up the maximum stain, and contains a small mass (or two or three masses) of solid birefringent concretions that appear very much like the ones seen by George (1936) in the blood cells of *Polyandrocarpa tincla*. When kept under continuous observation, these concretions are seen to mimic "Brownian movement," increase in size, and be evacuated into the cavity of the renal sac, while a fresh vacuole soon takes the place of the one discharged. These cells are therefore primarily instrumental not only in the absorption of excretory material from the body fluid bathing the outside of the sac, but also in the isolation and final deposition of solid excretory products into the renal sac. The cavity of the sac is filled with the transparent, yellowish-brown, renal fluid.

The renal concretions lie free in the cavity of the sac. They are located in concentrated clumps or masses more at the rounded end of the sac, and spread out in a more or less continuous chain, growing towards the tapering end of the sac. The

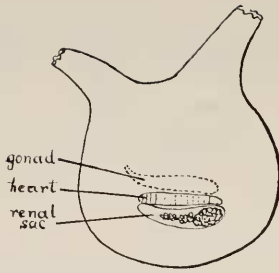


FIG. 1.

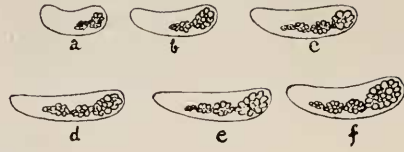


FIG. 2.

FIGURE 1. Diagrammatic sketch of right side of animal after removal of testis to show position of renal vesicle (bottom) in relation to pericardium (middle) and right gonad (top).

FIGURE 2. a-f: Stages in growth of renal sac and renal concretions, (a) being the youngest and (f) full grown.

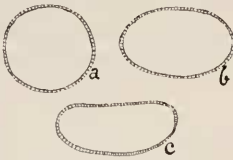


FIG. 3.

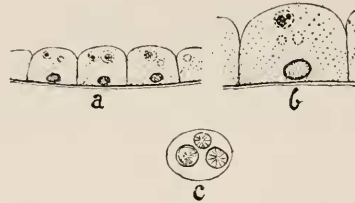


FIG. 4.

FIGURE 3. a and b: shape of fresh renal sac in T.S.; c: flattened outline after fixation.

FIGURE 4. a: the renal epithelium, $\times 120$; b: one renal epithelial cell showing a main vacuole containing concretions, and two secondary vacuoles being formed, $\times 250$; c: the main vacuole enlarged to show formative renal concretions, $\times 750$.

chain can be broken up into clumps even in the living renal sac, by merely shaking it. Each clump consists of a mass of discrete rounded or oval bodies, the birefringent granules, some of which are large and others small. The smallest ones measure about 3 to $4\ \mu$ across, while some of the largest ones were 30 to $40\ \mu$ in diameter. The larger granules show definite concentric lamellae or layers of growth, the central part of the granule showing radial striations. Two, three, or more con-

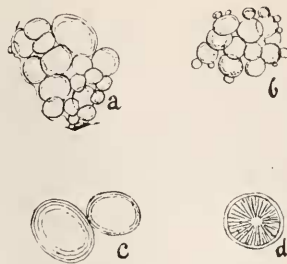


FIGURE 5. a and b: mass of renal concretions, showing attachment of later depositions on the previously deposited concretions in the renal sac; c: two concretion-bodies showing characteristic concentric layers; d: T.S. of a concretion-body showing radiating lines.

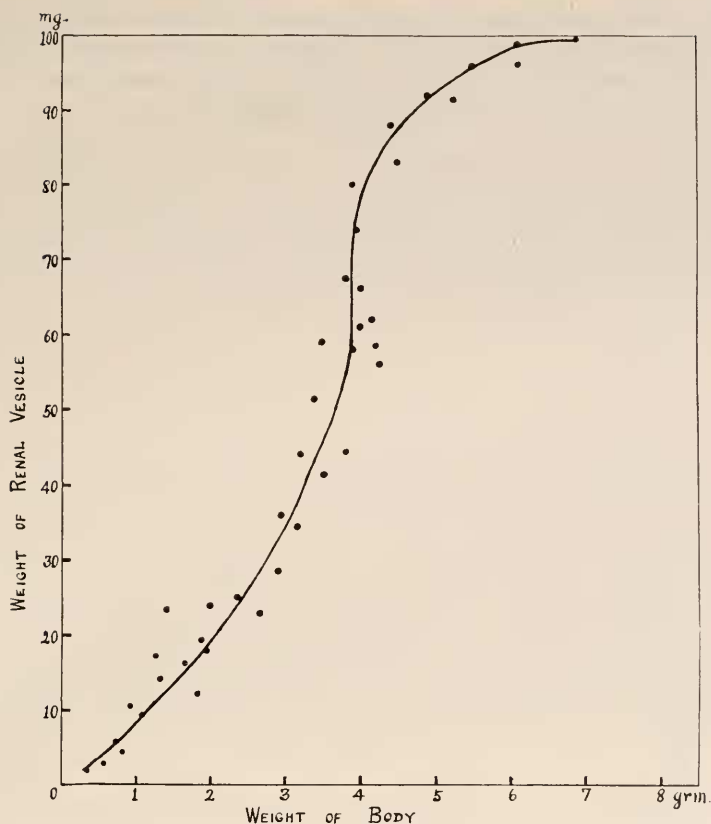


FIGURE 6. Correlation between body weight and weight of renal sac.

centric layers can be observed under the higher powers of the microscope (Fig. 5). This deposition clearly occurs from the renal fluid, without the wall of the sac taking any direct part in its formation. Smaller animals have definitely smaller excretory granules, while the largest ones show three, four, or more concentric lamellae. This appears to indicate that the rings or layers are formed by different rates of deposition of excretory matter, dependent on the differences in rates of metabolic activity at different periods in the life of the tunicate.

The neural gland opens by a neural duct into the branchial cavity, as in other tunicates. The cilia of the duct are long and lash away from the gland¹ towards

¹ Huus (1937) stated that "the ciliary pit whose function is not as yet known, may probably be an organ for the reception of sexual stimuli, and that the neural gland which is closely connected with the ciliary pit, responds to these stimuli with a hormone production which in turn calls forth spawning." Similarly, Butscher (1930), and Bacq and Florkin (1935) have shown that the neural gland in ascidians produces a hormone with similar physiological effects as pituitrin. Now a hormone is always produced by a ductless gland. The neural gland has not only a duct with cilia that beat outwards, but the entire central part of the gland consists of branching ductules joining to form the main duct. The outward beating cilia would not allow passage of fluids into the gland, although the dorsal tubercle itself may receive sexual stimuli. Pituitrin is probably produced by the peripheral part of N. gland.

the opening of the duct as seen in ducts dissected in Ringer's fluid. Cells bearing birefringent granules were seen to be extruded into the lumen of the gland and carried out by the "stream" produced by the cilia of the neural duct. This process is similar to the one observed in *Herdmania* (Das, 1936).

CORRELATION BETWEEN BODY WEIGHT AND WEIGHT OF RENAL SAC

Dahlgrün (1901), Azema (1926-29), and George (1936) state that waste matter is gradually deposited in the renal sac and stored in the form of concretions throughout life. They have not given any quantitative estimates of the concretions, nor

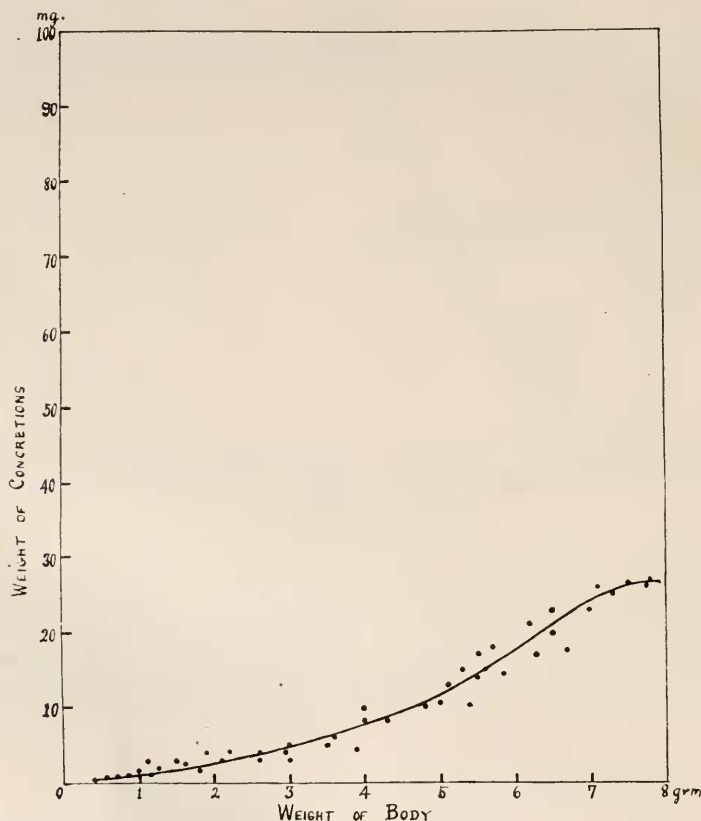


FIGURE 7. Correlation between body weight and weight of renal concretions.

have they established any correlations between the weight of concretions and the body weight, which alone would elucidate the rate and mode of disposal of excretory products. The present author isolated over one hundred renal sacs from animals of all possible sizes available at Woods Hole, ranging from a body weight of about half a gram to as large as seven grams. The weights of a series of individuals of graded sizes were taken and the weight of the renal sac of each was determined. The results are shown in graphical form in Figure 6. It is apparent from

the nature of the curve that the growth in weight of the renal sac is not directly proportionate to the growth in body weight. Up to a body weight of about three grams, the rate of growth of the sac is proportionate to that of the body; above this size, however, the rate of growth of the sac outstrips that of the body until a body weight of about five grams is reached. Thereafter the rate of growth of the sac becomes slower than that of the body, little growth taking place above a body weight of seven grams. This correlation may be interpreted as that between the body of the tunicate and the renal fluid, since the latter constitutes over 80 per cent by weight of the renal sac. It can thus be concluded that comparatively more renal fluid is present in the sac in half to full grown tunicates than in the younger or older stages. The correlation between body weight and weight of renal fluid is a dynamic one, as explained later.

CORRELATION BETWEEN BODY WEIGHT AND WEIGHT OF RENAL CONCRETIONS

If, as found by past workers on the subject, the solid concretions in the renal sac are stored throughout life, it was surmised that there should be a straight line relationship between the body weight and the weight of renal concretions in animals of different sizes. Actually, however, the data from the present study yield a curve which is far from a straight line. From a body weight of about 0.4 gm. up to about 3 gm., the increase in weight of the concretions is rather small; but above this the weight increases rapidly and the correlation curve shows a steep ascent (Fig. 7). This means that instead of the accumulation of excretion being proportional to the body weight (i.e. instead of the increase in excretory material being proportional to the growth of the animal), the excretory material is deposited at a faster rate as the animal grows older.

CORRELATION BETWEEN WEIGHT OF RENAL VESICLE AND WEIGHT OF RENAL CONCRETIONS

This correlation is shown in Figure 8. It appears to be almost a straight line relationship in its first half, meaning that the rate of accumulation of concretions is directly proportional to the increase in weight of the renal sac. In other words in the first part of the life of the tunicate, the increase of renal concretions is directly proportional to the increase in renal fluid inside the sac. As the tunicate grows older, however, the rate of increase of the renal sac is overtaken by the rate of deposition of renal concretions; and we find the upper part of the correlation line curving steeply upwards (Fig. 8). This increase falls rapidly as the maximum size of the tunicate is reached; and finally the weights of both the renal sac and the concretions remain almost stationary even with an increase in body weight. The final stage, when no further accumulation of concretions is possible, may be called the *saturation stage*. The tunicate could not live much longer once this stage has been reached.

The renal sac from large living *Molgula manhattensis* was removed and the animals kept under observation for a week or more, but no regeneration of the sac or formation of secondary renal sacs was observed. This is exactly the reverse of what obtains in multivesicled animals like *Ascidia* and *Asciidiella*, where a number of renal sacs are present instead of only one as in *Molgula*. In *A. mentula* (Azema, 1926) renal sacs are formed quite early in development; but instead of all the sacs

being formed simultaneously, they are formed successively in the regions around the oesophagus, stomach, and intestine. New vesicles arise as the old ones are used up. It is apparent, therefore, that these multivesicular tunicates have a more effective excretory system than the univesicular ones.

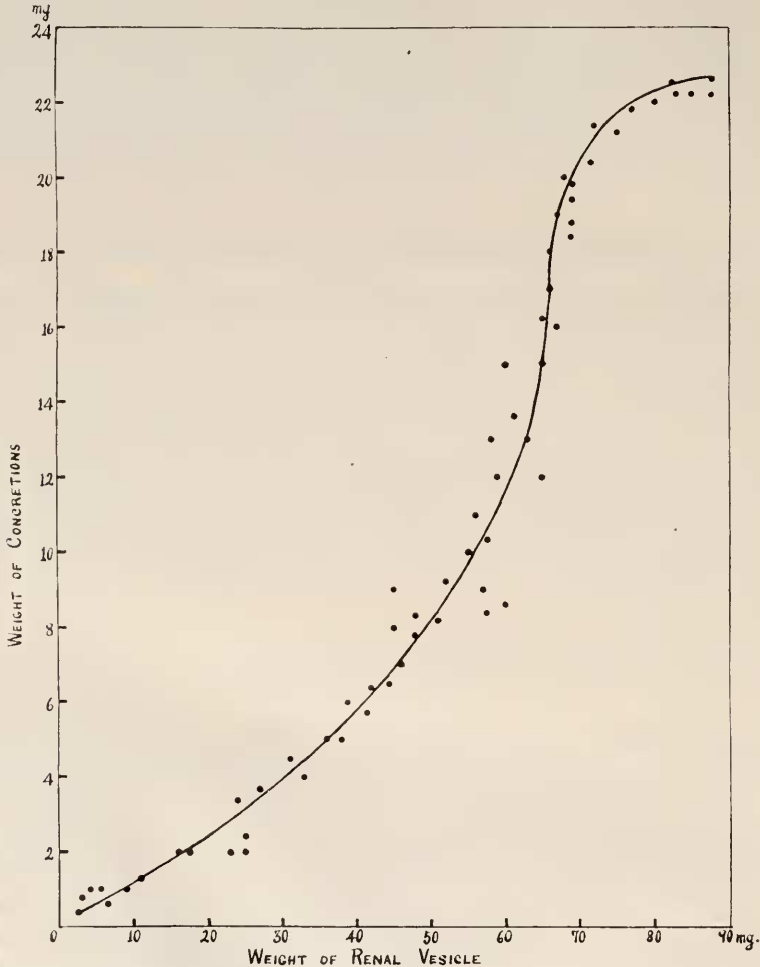


FIGURE 8. Correlation between weight of renal vesicle and weight of renal concretions.

THE RENAL FLUID

The renal fluid, when examined in fresh condition, is a clear, pale, yellowish-brown fluid which turns darker on exposure to air. The pH of this fluid ranged from 7.4 to 7.6 as determined by the Cambridge glass-electrode potentiometer. Tunicate urine is, therefore, slightly alkaline in nature. That this is caused by free ammonia was determined by the permutit method. No urea was present in any of the renal fluid samples. A full quantitative estimation of all the constituents of

the fluid could not be undertaken in the short time available at Woods Hole, but will no doubt be attempted in the near future.

Total molecular concentration and tonicity

Pure samples of the renal fluid were obtained from freshly dissected-out renal sacs by inserting a chemically clean, dry, glass micropipette into several sacs in turn. The pooled renal fluid was collected under mineral oil in order to avoid evaporation, absorption of water, or any changes due to exposure in air. The total molecular concentration of these fluids was compared with that of known NaCl solutions in "Barger tubes" (glass capillary tubes) using the method of Walker (1930 and 1937). This method involves sealing a series of micro-drops of the two fluids to be compared, each separated from its neighbours by a tiny air-gap, into chemically clean, dry, glass capillaries of very uniform internal diameter. Relative volumes of the drops are determined by measuring the lengths of the columns in the capillaries under a compound microscope, using a filar micrometer. These measurements are repeated at intervals for a period of two or three days, the capillaries being stored in a water bath between times. Consistent lengthening of a drop and shortening of its neighbours, indicates its higher total molecular concentration and vice versa. Thus by using saline solutions of known strength, the molecular concentration of the unknown fluid can be determined. Microdrops were prepared and read for:

- (a) renal fluid against known saline solution
- (b) body fluid against known saline solution
- (c) sea water against known saline solution
- (d) renal fluid against sea water
- (e) body fluid against sea water

The results may be summarized in tabular form:

SPECIMEN	APPROX. CORR. NaCl CONC.
Pooled body fluid	2.75 gm./100 cc.
Sea water	3.10 gm./100 cc.
Pooled renal fluid	3.45 gm./100 cc.
One sample of renal fluid	Hypertonic to sea water

This comparison of the total molecular concentration of the renal fluid, the sea water, and the body fluid, has, as will be presently seen, led to very interesting results. The renal fluid was thus found to be *hypertonic* to sea water in which the animal lives, while the body fluid was *hypotonic* to sea water, i.e.,

$$\text{renal fluid} > \text{sea water} > \text{body fluid.}$$

Osmoregulation

The occurrence of hypertonic urine in *Tunicata* throws new light on osmoregulation in this group, little work on this subject having been done in the past. The body of the tunicate is closed to all exchanges between the surface and the sea water due to the covering of the test. But the current of sea water passing through the branchial siphon into the pharynx and out through the atrial siphon, brings the extensive branchial wall and vessels into close contact with it. The O₂ is absorbed

and CO_2 is thus excreted through the branchial vessels. The body fluid, as established above, is *hypotonic* to sea water; and thus it is apparent that an osmotic gradient is maintained in the tunicate between the internal and the external environment. Whether water required by the animal is taken from the sea water into its alimentary canal like the marine teleostean fishes, is not definitely established but is quite probable. The role of the hypertonic renal fluid is, however, quite definite. There is a constant flow of fluids from the blood into the renal sac, the excretory products being extracted mainly by the cells constituting the wall of the sac. The renal cells not only maintain an active osmotic gradient, but allow more or less water to pass out of the sac according to the requirements of the animal. Experimental animals were kept in grades of sea water ranging from 40 per cent to 80 per

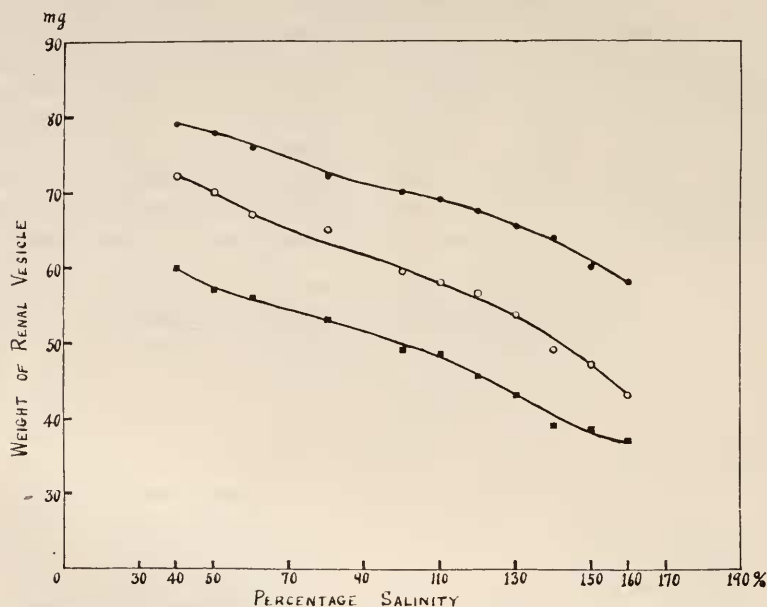


FIGURE 9. Correlation between weight of renal sac and different concentrations of sea water. Three experimental series are represented, the animals in each series weighing about the same.

cent (hypotonic), and from 110 per cent to 160 per cent (hypertonic), for a fixed period of five hours each. Each animal was weighed and a "twin" found weighing about the same. The "twins" were kept as controls in 100 per cent sea water for the same period. The renal sacs of each living experimental animal and its "twin" were rapidly dissected out and both weighed against each other. It was observed that renal vesicles from animals in diluted sea water weighed more than their controls, and that the extra weight was inversely proportional to the concentration of sea water in which the animals were kept; that is, the lower the concentration of sea water, the higher the weight of the renal sac. On the other hand, renal vesicles from animals kept in concentrated sea water weighed less than their controls; that is, the higher the concentration of sea water, the lower was the weight of the sac

(Fig. 9). It can therefore be concluded that the renal sac is an active osmoregulatory organ as well as an efficient extractor and storer of excretory material.

THE RENAL CONCRETIONS

As stated above, the concretions are dark brown in color, are birefringent in polarized light, and acquire a yellowish tinge by reflected light when spread out thinly on a glass slide. The concretions are insoluble in water and in dilute acids, but easily dissolved in diluted alkalis (NaOH, KOH). A qualitative analysis of the concretions was made to test for uric acid, xanthine, guanine, adenine, and creatinine. Folin's test yielded faint traces of creatinine; the murexide test and microscopic examination showed that a part of the concretions was made up of uric acid; Weidel's reaction indicated the presence of moderate amounts of xanthine; while the picrate test showed that even guanine was present. The zinc test for adenine was entirely negative, and the presence of hypoxanthine was also extremely doubtful. No allantoin could be traced in the concretions.

As shown in Figure 8, the rate of accumulation of concretions is directly proportional to increase in weight of the renal sac in the young tunicate. As it grows older, however, the rate of increase of the renal sac is overtaken by the rate of deposition of the concretions; and finally, when the saturation stage is reached, no more can be deposited.

THE MECHANISM OF EXCRETION

According to the findings of K  pffer (1872, 1874), Sulima (1914), Millot (1923), Schmidt (1924), and Azema (1926-1929), the main excretory products are: uric acid in some Ascidiidae, xanthine in Pyuridae, and un-named purine derivatives in other ascidians. This picture becomes more confusing if we now add guanine (found in Molgulidae) to this list. In all animals the ultimate source of the excretory nitrogen derivatives are the α -amino-N and the nucleoproteins. A list of the main nitrogen end products of various chordates may be given here:

sharks, dogfish—urea
bony fishes (Teolosts)—ammonia
frogs, newts—urea
turtles—urea
snakes, lizards—uric acid
birds—uric acid
mammals—urea

In tunicates, however, it appears that different families have, as the main nitrogen end product of excretion, either uric acid or one of the purine derivatives.

In chordates, where we find uric acid, urea, or ammonia as the main nitrogen end product, the conversions stop at these respective steps. In the tunicate, although some of the nitrogenous matter gets converted into uric acid, a large part of it appears to stay as xanthine and guanine. We thus find in *Tunicata* a unique case of incomplete conversion, which accounts for the presence of xanthine and guanine, as well as uric acid. The author hopes to make exact quantitative estimations of these three constituents, in different tunicates, in the near future.

SUMMARY

1. The present contribution includes a review of past work on excretion in *Tunicata*.

2. In *M. manhattensis* the single-celled wall of the renal sac absorbs the excretory products, develops the concretions, and discharges them into the cavity of the sac, where they are stored throughout life.

3. The concretions consist of granules, some of which show three or four concentric lamellae caused by further deposition of excretory matter on top of the originally secreted granule.

4. The correlation between body weight, weight of renal sac, and weight of concretions is given. It is shown that accumulation of concretions is not uniformly maintained throughout the life of the tunicate.

5. If the adult renal sac be entirely removed, no regeneration or formation of secondary sacs takes place.

6. The renal fluid is *hypertonic* to sea water, whereas the body fluid is *hypotonic*.

7. The molecular concentration of the renal fluid is 3.45 gm./100cc.; of the sea water at Woods Hole 3.10 gm./100 cc.; and of the body fluid 2.75 gm./100 cc., corresponding to NaCl concentrations.

8. The renal concretions contain xanthine, guanine, and uric acid.

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